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Gene expression pattern

Isolation of three zebrafish *dachshund* homologues and their expression in sensory organs, the central nervous system and pectoral fin buds

Katherine L. Hammond[a,b,*], Robert E. Hill[a], Tanya T. Whitfield[b], Peter D. Currie[a]

[a]*MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, UK*

[b]*Centre for Developmental Genetics, University of Sheffield School of Medicine and Biomedical Science, Firth Court, Western Bank, Sheffield, S10 2TN, UK*

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Abstract

Drosophila dachshund (*dac*) interacts with *sine oculis* (*so*), *eyes absent* (*eya*) and *eyeless* (*ey*) to control compound eye development. We have cloned three zebrafish *dac* homologues, *dachA*, *dachB* and *dachC*, which are expressed widely, in distinct but overlapping patterns. Expression of all three is found in sensory organs, the central nervous system and pectoral fin buds. *dachA* is also expressed strongly in the somites and *dachC* in the neural crest and pronephros. These expression domains overlap extensively with those of zebrafish *pax*, *eya* and *six* family members, the homologues of *Drosophila ey*, *eya* and *so*, respectively. This is consistent with the proposal that *Dach*, *Eya*, *Six* and *Pax* family members may form networks, similar to that found in the fly eye, in the development of many vertebrate organs.

Keywords: Eye; Ear; Otic vesicle; Sensory patch; Lateral line; Sensory ridge; Neuromast; Forebrain; Hypothalamus; Diencephalon; Midbrain; Habenula; Dorsal thalamus; Hindbrain; Rhombomere; Trigeminal ganglion; Spinal neuron; Spinal cord; Pronephros; Pectoral fin bud; Somite; Branchial arch; Neural crest; *dachshund*; *dac*; *dach*; *pax*; *eya*; *six*; Zebrafish

1. Results and discussion

Drosophila Dachshund (Dac) is fundamental to compound eye development and functions as part of an interacting network with Eyeless (Ey), Sine oculis (So) and Eyes absent (Eya; Treisman, 1999). Vertebrate homologues of *dac* have been isolated from mouse (*Dach1* and *Dach2*), human (*DACH1*) and chick (*Dach2*) and these are thought to act in networks similar to that found in the fly (Hammond et al., 1998; Caubit et al., 1999; Kozmik et al., 1999; Davis et al., 1999; Heanue et al., 1999; Davis et al., 2001). The vertebrate networks also include members of the *Pax*, *Eya* and *Six* gene families, the vertebrate counterparts of *ey*, *eya* and *so*, respectively.

In vertebrates, a *Pax/Eya/Six/Dach* network seems to be important in the development of a variety of organs, with a different selection of the *Dach*, *Pax*, *Eya* and *Six* family genes active in each tissue. Dach2, for instance, interacts with Pax3, Eya2 and Six1 in the developing chick somite (Heanue et al., 1999) and evidence is consistent with a similar network, including Dach1, Pax6, Eya1, 2, 3 and Six3, in the vertebrate eye (Hill et al., 1991; Xu et al., 1997; Loosli et al., 1999; Chow et al., 1999). Expression of members of all four gene families overlaps in many other areas including the pronephros, central nervous system (CNS), and otic vesicle, raising the possibility that a Dach/Eya/Pax/Six 'cassette' is important in all these tissues.

Here we report the isolation and characterization of three zebrafish *dachshund* genes, *dachA*, *dachB* and *dachC*. These genes are expressed extensively in the CNS, the pronephros and in the sensory organs of the lateral line, eye and ear, where many members of the zebrafish *pax*, *six* and *eya* families are also detected (Kobayashi et al., 2000; Nornes et al., 1998; Pfeffer et al., 1998; Riley et al., 1999; Sahly et al., 1999; Seo et al., 1998).

1.1. Isolation of dachA, dachB and dachC

We isolated *dachA*, *B*, and *C* by screening a zebrafish 15–19 h cDNA library with the dach-box N region (see below) of murine *Dach1*. These clones are 2883, 2357 and 2055 bp long, respectively and contain full length open reading frames of 602, 564 and 576 amino acids (Fig.

* Corresponding author. Centre for Developmental Genetics, University of Sheffield School of Medicine and Biomedical Science, Firth Court, Western Bank, Sheffield, S10 2TN, UK. Tel.: +44-114-222-2377; fax: +44-114-222-2788.

E-mail address: k.hammond@sheffield.ac.uk (K.L. Hammond).

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1a). DachC has the most similar amino acid sequence to murine Dach1, while DachA and DachB are more similar to chick and murine Dach2. Of the two, DachA is the most similar to Dach2 (Fig. 1b). Two regions highly conserved between mammalian and *Drosophila* Dachshund, Dach-box C and Dach-box N (also known as DD1 and DD2; Davis et al., 1999), are found in all three zebrafish proteins (Fig. 1b).

1.2. Expression analysis

We analyzed expression of *dachA*, *B* and *C* by whole-mount in situ hybridization between the 10 somite (s) stage and 72 h post-fertilization (hpf). All three genes are expressed widely, in distinct but overlapping patterns. *dachA* and *dachC* show significant expression similarity to mammalian *Dach1* and *Dach2*, but *dachB* is less similar, being predominantly expressed in the lateral line system, which has no mammalian counterpart (Hammond et al., 1998; Caubit et al., 1999; Kozmik et al., 1999; Davis et al., 1999, 2001).

1.2.1. Sense organs

1.2.1.1. Eye. Only *dachA* is expressed in the developing eye. It is detected throughout the optic vesicle by 12s, and then is restricted to the lens from 20s to 34 hpf (Fig. 2A). Lens expression disappears by 48 hpf when strong retinal expression is seen in the ganglion cell layer (Fig. 2B). Retinal expression remains weakly detectable at 72 hpf.

1.2.1.2. Ear. *dachA*, *dachB* and *dachC* are all expressed in the ear.

dachA is seen at 14s in two medial domains, one at each end of the otic vesicle (Fig. 2C). These are likely to be the areas where sensory hair cells of the maculae are developing (Haddon et al., 1998; Riley et al., 1997). From 20s (Fig. 2D,E) to 34 hpf (Fig. 2F), expression is seen in a dorso-medial band which encompasses the two original domains. In addition, at 34 hpf, expression is seen in the anterior macula and in an adjacent ventral epithelial region (Fig. 2F). At 48 hpf, weak expression is seen in both maculae. From 48 to 72 hpf, dorsal expression includes a duct-like

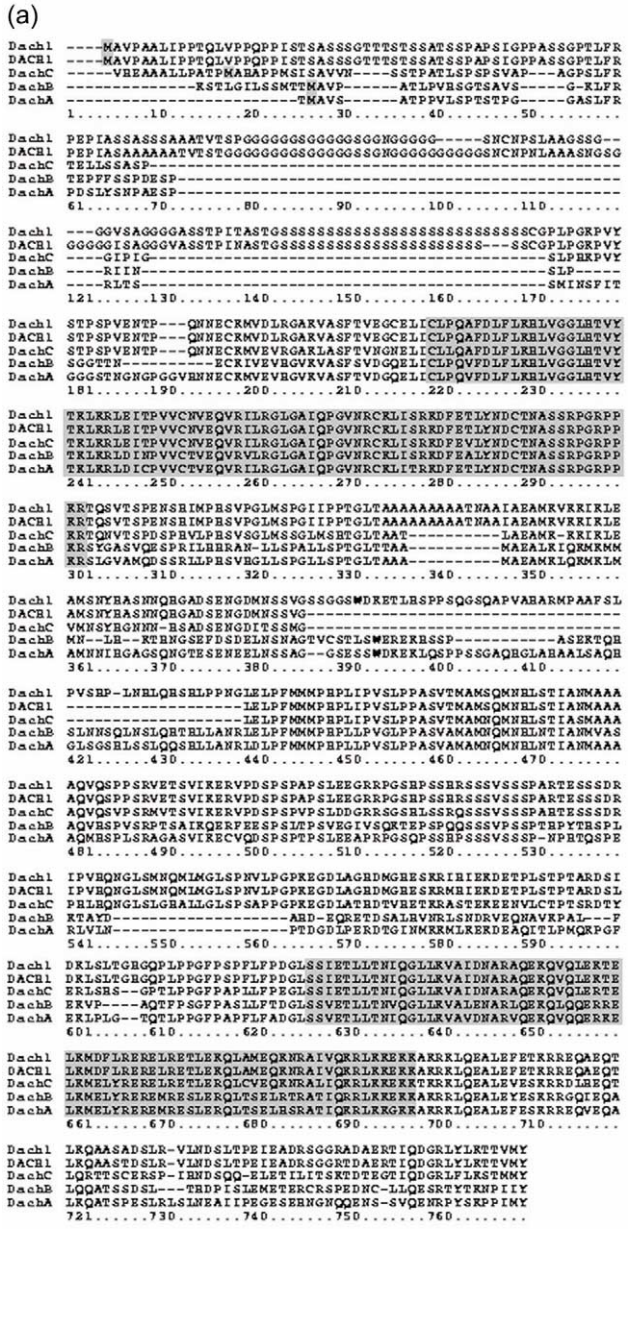

Fig. 1.

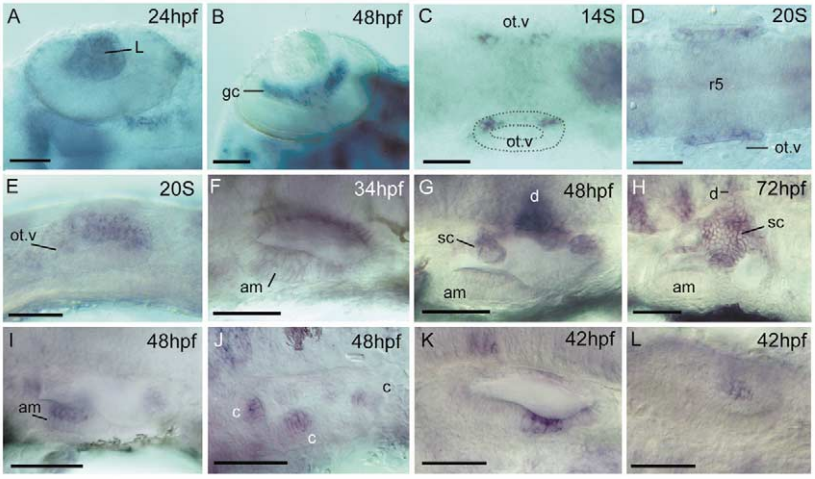

Fig. 2. Expression of *dachA*, *dachB* and *dachC* in the developing eye and ear. Anterior is to the left. (A–D) and (L) are dorsal views; all others are lateral views. Scale bar, 50 μm. (A) *dachA*, eye, 24 hpf, dorsal: expression is within the lens. (B) *dachA*, eye, 48 hpf, dorsal: expression is within the ganglion cell layer of the retina. (C) *dachA*, otic vesicle, 14s, dorsal: expression is located in two discrete medial regions, one at the anterior and one at the posterior end of the vesicle. One of the two otic vesicles is outlined for clarity. (D) *dachA*, otic vesicle, 20s, dorsal; (E) *dachA*, otic vesicle, 20s, lateral: a band of epithelial expression located dorsally and medially develops by 20s. Expression is similar at 24 hpf (not shown). Note the expression in odd numbered rhombomeres. (F) *dachA*, otic vesicle, 34 hpf, lateral: dorsal–medial epithelial expression remains. Ventral expression is now observed in the anterior macula and an adjacent region just posterior to this. (G) *dachA*, otic vesicle, 48 hpf, lateral: expression is in a dorsal epithelial duct-like structure which is likely to be an early endolymphatic duct, and in the projections at the anterior and posterior of the vesicle. These will form the semicircular canals. Weak expression remains in the anterior macula and the posterior macula (not shown). (H) *dachA*, otic vesicle, 72 hpf, lateral: expression is seen in the semicircular canal projections excluding the majority of the ventral projection. It remains in the dorsal duct as at 48 hpf. (I) *dachB*, otic vesicle, 48 hpf, lateral (focal plane at the level of the anterior macula): strong expression is seen in the hair cells of the anterior macula. The hair cells of the posterior macula (not shown) also express *dachB*. (J) *dachB*, otic vesicle, 48 hpf, lateral (lateral focal plane) strong expression is seen within the cristae. (K) *dachC*, otic vesicle, 42 hpf, lateral. (L) *dachC*, otic vesicle, 42 hpf, dorsal, lateral to top: expression is seen in a ventro-lateral region of epithelium. This expression begins by 34 hpf and disappears by 48 hpf (not shown). L, lens; am, anterior macula; gc, ganglion cell layer; ot.v, otic vesicle; r5, rhombomere 5; c, crista; sc, semicircular canal projection; d, dorsal duct-like structure.

structure, believed to be the presumptive endolymphatic duct (Fig. 2G,H). Expression is also detected in the semicircular canal projections (Fig. 2G,H).

dachB is expressed in the maculae at 34 hpf and in all sensory patches from 48 to 72 hpf (Fig. 2I,J).

dachC is expressed in a ventral epithelial region from 34 to 42 hpf (Fig. 2K,L).

1.2.1.3. Lateral line system. Only *dachB* is strongly expressed in the lateral line system, although *dachA* is

Fig. 1. (a) Amino acid alignment of the predicted DachA, DachB and DachC open reading frames (ORFs) with murine Dach1 and human DACH1 protein sequences (accession numbers AAF102547 and AAF102546, respectively). Dach-box N (numbered 220–302) and Dach-box C (numbered 627–698), two regions of high homology between all known *dac* homologues, are boxed in grey (shown in detail in (b)). All three zebrafish clones are predicted to contain full length ORFs beginning at the boxed methionine; *dachA* and *dachB* have 156 and 119 bp of sequence, respectively, upstream of the proposed initiating methionine, each containing two in-frame stop codons (amino acid sequence between the more 3′ stop codon and the initiating methionine is shown). DachC is presumed to be full length as the proposed ORF is similar in length to the DachA and DachB ORFs and an alanine residue following the initiating methionine of DachC is conserved with DachA, DachB and mammalian Dach1 and DACH1. There are, however, no upstream in-frame stop codons in the 36 bp of sequence upstream of the proposed DachC initiating methionine (A translation of all upstream DachC sequence is shown). The DachA, DachB and DachC ORFs are 602, 564 and 576 residues, respectively. These are shorter than human and murine Dachshund proteins, which contain 754 and 702 amino acids, respectively. The DachA and Dach1 clones contain an insertion of 52 amino acids (numbered 387–439), which is partially present in DachB but absent from DachC and human DACH1. This probably corresponds to an alternatively spliced exon (RT-PCR analysis of mouse complete cDNA confirms that both splice forms are present in this organism and that the insertion is a rare alternative splice form; Hammond et al., 1998). A further potential alternative splice region is present in Dach1, DACH1 and DachC but absent from DachA and DachB (numbered 546–564). Several repetitive regions present in the mammalian genes are absent from all three zebrafish genes. (b) Consensus amino acid alignment of two highly conserved regions found in all known Dachshund homologues. Dach-box N (also known as DD1: Davis et al., 1999) spans 83 amino acids near the N-terminus of Dachshund. Identity of DachA, DachB and DachC is 94, 93 and 98%, respectively with murine Dach1 and 96, 94 and 89% with murine Dach2 within this region (excluding the insertion apparently only found in Dach2). Dach-box C (also known as DD2: Davis et al., 1999) spans 72 amino acids near the C-terminus of Dachshund. Identity of DachA, DachB and DachC, respectively is 72, 68, and 87% with murine Dach1 and 79, 76 and 71% with mouse Dach2 in this region. Based on these amino acid sequence comparisons, it appears that DachC is most similar in sequence to murine Dach1 and that DachA and DachB are more similar to murine Dach2. Black, conserved residues; grey, similar residues; white, non-conserved residues; zebA, DachA (zebrafish); zebB, DachB (zebrafish); zebC, DachC (zebrafish); mouse1, Dach1 (mouse; Hammond et al., 1998; Caubit et al., 1999; Davis et al., 1999; Kozmik et al., 1999); human1, DACH1 (human; Hammond et al., 1998; Caubit et al., 1999; Davis et al., 1999; Kozmik et al., 1999); chick2, Dach2 (chick; Heanue et al., 1999); mouse2, Dach2 (mouse; Davis et al., 2001); Dros, dac (*Drosophila*; splice variant 4 accession number U19269; Mardon et al., 1994); C.elegans, *Caenorhabditis elegans* Dachshund homologue (cosmid B401).

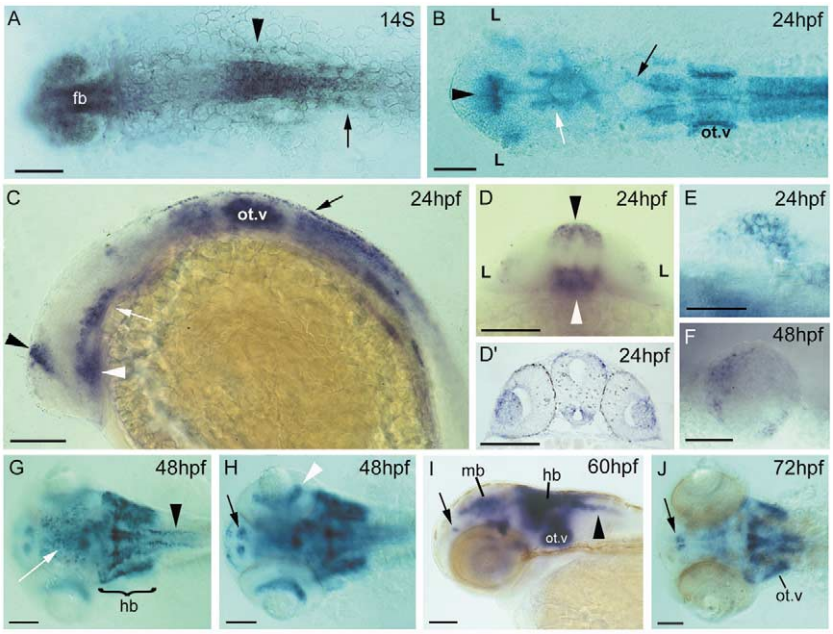

Fig. 3. Expression of *dachA* in 14s to 72 hpf embryos. Anterior is to the left, except (D) which is an optical cross-section (dorsal to top) and (D′) which is a transverse section (dorsal to top), both at the level of the arrowheads in (C). Scale bar, 100 μm, except E/F where it is 50 μm. (A) 14s, dorsal view: expression is seen within the forebrain (fb), eye, spinal cord, lateral mesoderm (black arrowhead) and the first 4–5 somites (black arrow). (B) 24 hpf, dorsal view; (C), 24 hpf, lateral view; (D), 24 hpf frontal view; (D′), 24 hpf transverse section at the level of arrowheads in (C): expression is seen in the dorsal forebrain (black arrow head), hypothalamus (white arrow head), neural crest (black arrow), lens (L), otic vesicle (ot.v.), rhombomeres 3, 5 and 7 of the hindbrain, lateral mesoderm, nucleus of the medial longitudinal fasciculus (white arrow) and in the anterior spinal cord. Expression at 20s (not shown) is similar to 24 hpf. (E) Pectoral fin bud, 24 hpf, dorsal view (distal to top); (F), pectoral fin bud, 48 hpf, lateral view (distal to top): expression is seen throughout most of the bud at 24 hpf, tending towards the posterior; by 48 hpf, a small region of expression is seen at the posterior and a larger region at the anterior. This resembles the pattern of murine *Dach1* in the limb bud at E10.5–11.5 (Hammond et al., 1998). (G) 48 hpf, dorsal view (dorsal focal plane); (H), 48 hpf, dorsal view (more ventral focal plane): expression is seen in the ganglion cell layer of the eye (white arrow head), the hindbrain (hb), the anterior spinal cord (black arrowhead), the midbrain including the tectum (white arrow) and in the forebrain (black arrow). (I) 60 hpf, lateral view: expression is similar to that at 48 and 72 hpf. It is detected in the hindbrain (hb), anterior spinal cord (black arrowhead), forebrain (black arrow), midbrain (mb) and in the otic vesicle (ot.v). Weak expression remains visible in the ganglion cell layer of the eye (slightly out of focal plane). (J) 72 hpf, dorsal view: expression remains in the hindbrain, forebrain (black arrow), otic vesicle (ot.v) and more weakly in the midbrain.

expressed weakly in the migrating midbody lateral line primordium and neuromasts (not shown).

dachB is expressed in the pre-otic lateral line placode from 15s (Fig. 4B,E—shown at 20s) and continues to be expressed in the sensory ridges (Fig. 4D) and, until 72 hpf, in the neuromasts derived from these (Fig. 4G,H). The midbody lateral line primordium expresses *dachB* strongly as it migrates the length of the body from 20s until 48 hpf (Fig. 4C,F—shown at 24 hpf). The neuromasts it deposits also express *dachB* until 72 hpf (data not shown).

1.2.2. CNS

All three fish genes are expressed in the CNS.

1.2.2.1. dachA. *dachA* is first detected in early somitogenesis in the forebrain and spinal cord (Fig. 3A–shown at 14s). Hindbrain expression, in rhombomeres 3, 5, and 7, is seen at 16s and is maintained until 34 hpf (Fig. 3B,C). By 48 hpf, hindbrain expression remains but is not obviously restricted to specific rhombomeres (Fig. 3G). Forebrain expression is seen in a dorsal region and in the hypothalamus by 20s (Fig. 3B–D—shown at 24 hpf); the nucleus of the medial longitudinal fasciculus expresses *dachA* at 24 hpf (Fig. 3B,C). By 48 hpf, complex midbrain expression appears and forebrain and anterior spinal cord expression remain (Fig. 3G–I). Forebrain, midbrain and hindbrain expression is still present at 72 hpf (Fig. 3J).

1.2.2.2. dachB. At 10s, ventro-medial expression is seen in the spinal cord and lateral, punctate expression, representing individual neurons, is seen in the rostral embryo (Fig. 4A). At 20s, punctate expression throughout the caudal neural tube is seen but ventro-medial expression has disappeared (Fig. 4B,E). At 24 hpf, the hindbrain and the nucleus of the post-optic commissure express *dachB* (Fig. 4C,F) and at 48 hpf, expression is detected in the hindbrain and neurons of the midbrain tegmentum (Fig. 4G–I).

1.2.2.3. dachC. Forebrain expression begins to appear at 20s and is strong in the diencephalon by 24 hpf (Fig. 5B,C). Expression is now also seen in the cerebellum, rhombomere 4 of the hindbrain and spinal neurons (Fig. 5B,C). By 34 hpf, the trigeminal ganglion expresses

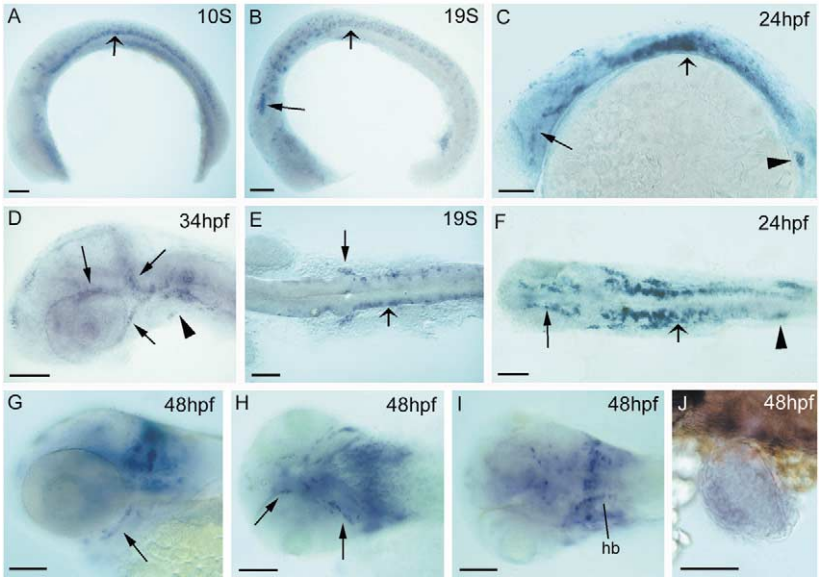

Fig. 4. Expression of *dachB* in 10s to 48 hpf zebrafish embryos. Anterior is to the left. Scale bar, 100 μm, except in (J) where it is 50 μm. (A) 10s, lateral view: expression is seen in a ventro-medial domain of spinal cord (wide arrow) and in individual, lateral neurons in the rostral part of the embryo. (B) 19s, lateral view; (E), 19s, dorsal view: expression is seen in individual neurons throughout the neural tube and in the pre-otic lateral line placode (thin arrow) which is seen just beginning to split to form dorsal and ventral sensory ridges. Ventro-medial expression has now disappeared (wide arrow). (C) 24 hpf, lateral view; (F), 24 hpf, dorsal view: expression remains in discrete, lateral regions of the hindbrain and anterior spinal cord (wide arrow) and is seen in the migrating midbody lateral line primordium (arrowhead) and the nucleus of the post-optic commissure (thin arrow). (D) 34 hpf, lateral view: the three sensory ridges (arrows) derived from the pre-otic lateral line placode all express *dachB*. Expression is also seen in one branchial arch (arrowhead). (G) 48 hpf, lateral view; (H), 48 hpf, dorsal view; (I), 48 hpf dorsal view: expression is seen in the nascent neuromasts deposited around the periphery of the eye by the sensory ridges (thin arrows (G,H)), the hindbrain (hb) and in individual neurons of the midbrain tegmentum (I). (J) 48 hpf, pectoral fin bud, dorsal view (distal to bottom): expression is seen weakly in the developing muscle masses.

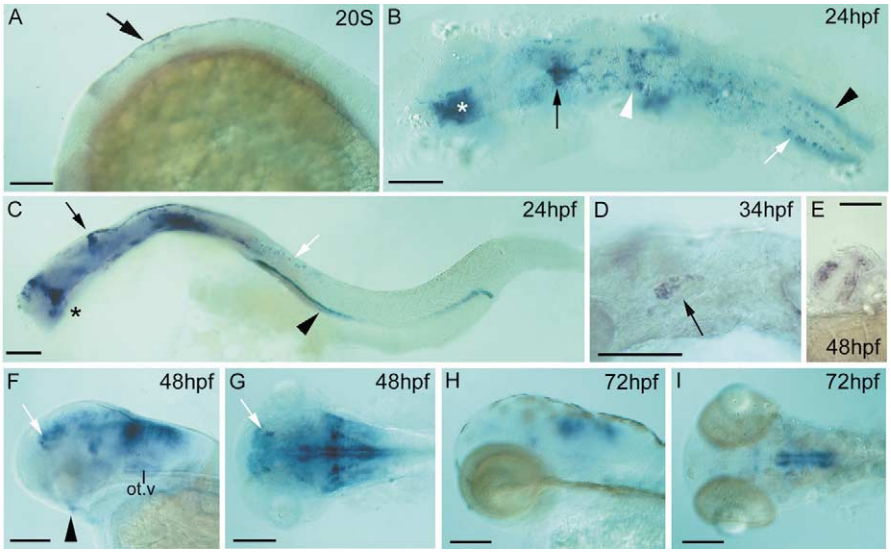

Fig. 5. Expression of *dachC* in 20s to 72 hpf zebrafish embryos. Anterior is to the left. Scale bar, 100 μm, except (E) where it is 50 μm. (A) 20s, lateral view (dorsal to top): expression is strong in the neural crest, seen migrating ventrally (black arrow). At 10–15s expression is similar although migration has yet to occur (not shown). This is very similar to murine *Dach1* expression, which is seen in premigratory and migratory neural crest. (B) 24 hpf, dorsal view; (C), 24 hpf, lateral view (dorsal to top): expression is seen in the cerebellum (black arrow), pronephric duct (black arrowhead), spinal neurons (white arrow), neural crest, diencephalon (*) and deep within rhombomere 4 of the hindbrain (white arrowhead). Just posterior to rhombomere 4 (below focal plane), branchial arch expression is seen. (D) 34 hpf, lateral view: shows trigeminal ganglion expression (black arrow) between the eye and the ear. (E) Pectoral fin bud, 48 hpf, dorsal view (distal to top): The developing muscles express *dachC* strongly at 48 hpf. (F) 48 hpf, lateral view; (G), 48 hpf dorsal view: expression is seen in the habenula and dorsal thalamus (white arrow), rhombomeres of the hindbrain, and in the midbrain tectum and tegmentum. (H) 72 hpf, lateral view; (I), 72 hpf, dorsal view: expression is mainly restricted to two longitudinal hindbrain regions either side of the midline.

dachC (Fig. 5D). At 48 hpf, *dachC* is seen in the habenula, dorsal thalamus, midbrain tectum and tegmentum, cerebellum and rhombomeres of the hindbrain (Fig. 5F,G). At 72 hpf, expression is mainly restricted to two longitudinal hindbrain stripes (Fig. 5H,I).

1.2.3. Other domains of expression

1.2.3.1. Pectoral fin buds. All three *dach* genes are expressed in the pectoral fin buds. *dachA* is seen throughout the bud at 24 hpf but is restricted to a large anterior and smaller posterior domain by 48 hpf (Fig. 3E,F). *dachB* and *dachC* are both found in the distal bud at 30 hpf. *dachC* is strongly expressed in the developing fin muscle at 48 hpf (Fig. 5E), while *dachB* is weakly expressed in a similar region (Fig. 4J). A small ventral region between the pectoral fin buds expresses all three genes strongly at 24 hpf (data not shown).

1.2.3.2. Somites. dachA is expressed transiently in the most anterior 4–5 somites from 12s to 16s (Fig. 3A).

1.2.3.3. Branchial arches. dachB is expressed in a single branchial arch at 34 hpf (Fig. 4D) and *dachC* is expressed in two at 24 hpf (Fig. 5B).

1.2.3.4. Pronephros. dachC is strongly expressed in the pronephros at 24 hpf (Fig. 5B,C).

1.2.3.5. Neural crest. dachC is expressed strongly in the premigratory and migratory neural crest from 10s until at least 24 hpf (Fig. 5A–C). *dachA* can also be seen here at 24 hpf (Fig. 3B,C)

2. Materials and methods

dachA was isolated by screening a zebrafish 15–19 h (28.5 °C) polyA$^+$ cDNA library in Uni-Zap XR lambda vector (a gift from Bruce Appel, University of Oregon) with a template produced by PCR amplification of the Dach-box N conserved region of murine *Dach1*, using primers GCT TTC GAC CTG TTC CTG AAG and CTT TGA GTC CTC TTA GGA GGC. Hybridization was carried out as in Nehls et al. (1994), after which positive clones were excised from the Uni-Zap phage vector (Stratagene) to produce pBluescript SK + /− phagemid.

dachB and *dachC* were isolated by re-screening the library with probe amplified from the Dach-box N region of *dachA* using primers AAC TGG CAT TGG TGC AGT CG and GTG AAA GTG GCC TCG TTC AC.

Sequencing was carried out using an ABI 337A automated fluorescent sequencer and an ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (Perkin–Elmer Applied Biosystems). Sequences were analyzed using Applied Biosystems 377A software and programs available through http://workbench.sdsc.edu.

2.1. In situ hybridization

Whole-mount in situ hybridization was carried out as in Oxtoby and Jowett (1993). Probes were labelled using a DIG labelling kit (Roche). Embryos used were either WIK or *gol*$^{-/-}$ and some embryos were treated with 0.0035% 1-phenyl-2-thiourea (Sigma), to prevent pigment formation (Westerfield, 1994).

For microscopy, embryos were cleared and mounted in glycerol or were dehydrated through an ethanol series, cleared in 2:1 benzyl alcohol/benzyl benzoate, and mounted in DePeX (BDH).

3. Note

Sequence data for *dachA*, *dachB* and *dachC* have been deposited with GenBank, accession numbers AF427108, AF427109 and AF207110.

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